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In re PATENT APPLICATION of:

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For: LIPID METABOLISM IMPROVING AGENT

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DECLARATION PURSUANT TO 37 C.F.R. 1.132

Sir:

I, Satoshi Nagaoka, of Gifu-Shi, Gifu 501-1193, Japan hereby declare as follows,

I graduated from Department of Agricultural Chemistry, Faculty of Agriculture, Utsunomiya University in March, 1981,and graduated from Department of Agricultural Chemistry, Faculty of Agriculture, Nagoya University in March, 1986, and got a doctor's degree in nutritional biochemistry for a thesis entitled "Comparative studies on the hypercholesterolemia induced by dietary xenobiotics or dietary excess tyrosine in rats." from Nagoya University in April, 1987.

Research Experience:

1988-1989 Nagoya University, Japan, Awardee of the Fellowships of Japan Society for the Promotion of Science for Japanese Junior Scientists, Nutritional

Biochemistry

1989-1993 Gifu University, Research Assistant, Nutritional Biochemistry

1994-1995 Boston University, School of Medicine, USA, Overseas Research Scholar (Ministry of Education), Biochemistry, Molecular Genetics

1993- Gifu University, Faculty of Agriculture, Japan, Associate Professor Nutritional Biochemistry

1996 Awardee of the Encouragement Award of the Japanese Society of Nutrition and Food Science

I am one of the co-inventors of the invention described and claimed in the application and have full knowledge of the present invention and cited references.

I conducted the following experiments to examine the effect of protein/phospholipid complex or protein hydrolyzate/phospholipid complex in improving lipid metabolism.

Experiment I

[Materials and methods]

Preparation of soy protein/phospholipid complex

As a phospholipid source, enzymatically decomposed phospholipid (EDPL)(Elmizer AC; T&K lecithin, Mie, Japan) which was prepared by hydrolyzing soy phospholipid with phospholipase A_2 was used.

Soy protein (New Fujipro-E; Fuji Oil, Osaka, Japan) was dispersed in water

and then stirred at 10,000 rpm for 5 minutes to make a solution. EDPL was then added to the solution such that the ratio of soy protein and EDPL was 4:1. The mixture was stirred at 10,000 rpm for 5 minutes to prepare a solution containing soy protein/EDPL complex. The solution was freeze-dried to make a product SP.

Separately, to the soy protein/EDPL complex solution, pepsin (activity; 1:10,000, Nacarai tesque, Kyoto, Japan) was added at 1% (w/w) at pH2, and the solution was incubated at 37°C for 24 hours. The reaction was stopped by heating at 90°C for 30minutes, and the mixture was neutralized with 2M NaOH. After diluting the solution with three volumes of water, the solution was centrifuged at 10,000 × g for 10minutes and the precipitate was recovered. The precipitate was freeze-dried to obtain an undigested high molecular weight fraction of the soy protein/EDPL complex (SPHP).

The components of EDPL were as follows (g/100g): phosphatidylcholine, 8.1; phosphatidylethanolamine, 5.4; phosphatidylinositol, 13.2; phosphatidic acid, 4.9; lysophosphatidylcholine, 25.8; lysophosphatidic acid, 8.4; phytic acid, 7.0; glycerophosphorylethanolamine, 15.4; glycerophosphoinositol, 3.5; triglyceride, 3.0; water, 0.5; lysophosphatidylethanolamine, 0.1; lysophosphatidylinositol, 0.1.

Animals and diets

Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) weighing about 90g were used. Room temperature was maintained at 22 ± 2°C with a 12 hour cycle of light (8:00-20:00) and darkness. All the rats were housed individually

in metal cages and were allowed free access to diets and water. After acclimation to a commercial stock diet (CE-2; Japan CLEA, Tokyo) for 3days, rats were divided into groups on the basis of body weight. The composition of the basal diet, as recommended by the American Institute of Nutrition (1977), was shown in Table 1.

Table 1. Composition of the diets (g/kg)

	Soyprotein	SP	SPHP
Soyprotein	230.7		
SP		301.2	
SPHP			374.5
Lard	50	50	50
Corn oil	10	10	10
Mineral mixture*	35	35	35
Vitamin mixture*	10	10	10
Choline chloride	2	2	2
Sucrose	654.8	584.3	511.0
Cholesterol	5	5	5
Sodium cholate	2.5	2.5	2.5

SP, soyprotein with bound phospholipids; SPHP, soyprotein peptic hydrolyzate with bound phospholipids.
* AIN-76 diet (American Institute of Nutrition, 1977).

The purity of each protein was as follows: soy protein (867.0g/kg), SP (664.0g/kg), SPHP (534.0g/kg). The lipid content of each protein was as follows: soy protein (15.0g/kg), SP (215.0g/kg), SPHP (392.0g/kg).

Rats were divided into 3 groups of 6 rats on the basis of body weight. Each group was fed freely one of the respective test diets containing soy protein, SP and SPHP as the protein source for 10days. Each rat was measured its body

weight everyday throughout this experiment with an electronic balance. After fasting for 24 hours, the rats were anesthetized with diethyl ether. Whole liver was excised from each rat body. Each liver was weighed with an electronic balance after rinsed with ice-cold saline.

Lipid analysis

Liver lipids were extracted with chloroform-methanol (2:1, v/v) in accordance with Folch partition method, and total lipids were determined gravimetrically.

Lipid contents were determined by use of commercially available kits as follows: liver cholesterol with Monotest cholesterol (Boehringer Mannheim Yamanouchi, Tokyo, Japan; liver triacylglycerol with Triglycolor III (Boehringer Mannheim Yamanouchi, Tokyo, Japan.

Statistical analysis

Results are expressed as means with SEM. The statistical significance of differences was evaluated by Duncan's multiple-range test after one-way ANOVA. The significance levels quoted are two-sided. Results were considered significant at $P<0.05$.

[Results]

The results are shown in Table 2.

Table 2. Body weight gain, food intake, liver weight, liver lipids in rats fed diets with soyprotein, SP and SPHP *†

(Mean values with standard errors of mean for six rats)

	Soyprotein		SP		SPHP	
	Mean	SEM	Mean	SEM	Mean	SEM
Body weight gain, g/10d	23 9 a	1.3	24.1 a	1.7	22 6 a	1.3
Food intake, g/day	14 8 ab	0.6	14.9 b	0 7	13 0 a	0 6
Liver weight, g/100g body weight	4 01 a	0.18	3.91 a	0.14	3 85 a	0 09
Liver,						
Total lipids, mg/g liver	137 3 c	2.6	98.5 b	3 4	58 1 a	1.3
Cholesterol, μmol/g liver	65 0 c	3.2	39.0 b	2.1	10 0 a	0.4
Triacylglycerol, μmol/g liver	28 6 b	1.1	23.2 b	1.6	9 7 a	1 0
Phospholipid, μmol/g liver	112 4 c	3 7	81.5 b	4.0	59 0 a	1 1

Soyprotein, 20% soyprotein; SP, 20% soyprotein with bound phospholipids; SPHP, 20% soyprotein peptic hydrolyzate with bound phospholipids.
* For details of diets, see Table 1.
† Mean values within a row not sharing a common letter are significantly different, $P<0.05$ (Duncan's multiple-range test).

[Conclusion]

As shown in Table 2, there were no significant differences in body weight gain and food intake among the group, but contents of total-lipids, cholesterol, triacylglycerol and phospholipid in the liver of SP fed group and in particular SPHP fed group were lower than those of soy protein fed group.

Experiment II

[Materials and methods]

Preparation of soy protein hydrolyzate/phospholipid complex

As a phospholipid source, enzymatically decomposed phospholipid

(EDPL)(Elmizer AC; T&K lecithin, Mie, Japan was used.

The soy protein/EDPL complex (SPHP) was prepared by the same method as described in Experiment I.

Animals and diets

Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) weighing about 90g were used. Room temperature was maintained at 22 ± 2°C with a 12 hour cycle of light (8:00-20:00) and darkness. All the rats were housed individually in metal cages and were allowed free access to diets and water. After acclimation to a commercial stock diet (CE-2; Japan CLEA, Tokyo) for 3days, rats were divided into groups on the basis of body weight. The composition of the basal diet, as recommended by the American Institute of Nutrition (1977), was shown in Table 3. Methionine, an essential amino acid, was added into the basal diet in order to make the nutritive values of both composition equal.

Table 3. Composition of the diets (g/kg)

	Soyprotein	SPHP
Soyprotein	235.3	
SPHP		353.4
Lard	50	50
Corn oil	10	10
Mineral mixture*	35	35
Vitamin mixture*	10	10
Choline chloride	2	2
Lactose	3.5	3.5
Sucrose	198.7	159.5
Celllose	50	50
Cholesterol	5	5
Sodium cholate	2.5	2.5
Corn Starch	397.4	318.9
Methionine	0.9	0

SPHP, soyprotein peptic hydrolyzate with bound phospholipids.
* AIN-76 diet (American Institute of Nutrition, 1977).

Rats were divided into 2 groups of 6 rats on the basis of body weight. Each group was fed freely one of the respective test diets containing soy protein and SPHP as the protein source for 10days. Each rat was measured its body weight everyday throughout this experiment with an electronic balance. After fasting for 24 hours, the rats were anesthetized with diethyl ether. Blood was collected from each rat by cardiac puncture. Whole liver was excised from each rat body. Each liver was weighed with an electronic balance after rinsed with ice-cold saline.

Lipid analysis

Serum was separated from blood with a centrifuge. Liver lipids were extracted with chloroform-methanol (2:1, v/v) in accordance with Folch partition

method, and total lipids were determined gravimetrically.

Lipid contents were determined by use of commercially available kits as follows: serum and liver cholesterol with Monotest cholesterol (Boehringer Mannheim Yamanouchi, Tokyo, Japan; serum and liver triacylglycerol with Triglycolor III (Boehringer Mannheim Yamanouchi, Tokyo, Japan.

Statistical analysis

Results are expressed as means with SEM. The statistical significance of differences was evaluated by Student's t-test. The significance levels quoted are two-sided. Results were considered significant at $P<0.05$.

[Results]

The results are shown in Table 4.

Table 4. Serum and liver lipids in rats fed diets with soyprotein and SPHP

(Mean values with standard errors of mean for six rats)

	Soyprotein		SPHP	
	Mean	SEM	Mean	SEM
Serum,				
Cholesterol, mg/dl	112.8	21.7	79.0 **	12.9
Triacilglycerol, mg/dl	101.8	26.9	89.8	10.0
Liver,				
Cholesterol, μmol/g liver	36.6	1.9	4.1 ***	0.5
Triacylglycerol, μmol/g liver	42.7	5.3	14.4 ***	1.2

Soyprotein, 20% soyprotein; SPHP, 20% soyprotein peptic hydrolyzate with bound phospholipids.
For details of diets, see Table 1.
Astarisks represent the significant difference from soyprotein group using Student's t-test (**, $p<0.01$; ***, $p<0.001$).

[Conclusion]

As shown in Table 4, contents of cholesterol and triacylglycerol in the serum and liver of SPHP fed group were lower than those of soy protein fed group.

On the other hand, there were no significant differences in body weight gain and food intake among the group.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: This 9th day of March, 2002.

Satoshi Nagaoka

Satoshi Nagaoka